Tests of associational defence provided by hairy plants for glabrous plants of Arabidopsis halleri subsp. gemmifera against insect herbivores

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Tests of associational defence provided by hairy plants for glabrous plants of *Arabidopsis halleri* subsp. *gemmifera* against insect herbivores

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Abstract

1. We investigated trichome-producing (hairy) and trichomeless (glabrous) plants of *Arabidopsis halleri* subsp. *gemmifera* to test whether plant resistance to herbivory depends on the plants' phenotypes and/or the phenotypes of neighbouring plants (associational effects).

2. We conducted a common garden experiment in which the relative frequency of hairy and glabrous plants was manipulated. Two species of leaf-chewing insects (larvae of a white butterfly and a cabbage sawfly) were found less often on hairy plants than on glabrous plants. In contrast, the numbers of aphids and flea beetles did not differ significantly between hairy and glabrous plants. For none of these insects did abundance depend on the frequency of the two plant morphs.

3. A field survey was conducted in two natural populations of *A. halleri*. In the first population, a species of white butterfly was the dominant herbivore, and hairy plants incurred less leaf damage than glabrous plants across two years. In contrast, in the other population, where flea beetles were dominant, there were no consistent differences in leaf damage between the two types of plants. In neither of the two populations did we find evidence for associational effects.

4. This study did not provide any conclusive evidence of associational effects of anti-herbivore resistance, but we did find that trichomes can confer resistance to certain herbivores. Given the results of our previous work on associational effects against a flightless leaf beetle, such associational effects of the trichome dimorphism of *A. halleri* were herbivore-specific. [243 up to 250 words]

Key words: Anti-herbivore resistance, *Arabidopsis halleri* subsp. *gemmifera*, Associational effects, Polymorphism, Trichome

Introduction

As sessile organisms, plants are consumed by a diverse array of insect herbivores (Strong *et al*., 1984; Lewinsohn *et al.*, 2005). Thus, plants developed various resistance traits to protect themselves from herbivory (Schoonhoven *et al.*, 2005). For example, trichomes (plant epidermal hairs) are considered a physical resistance trait against herbivory (Levin, 1973; Hanley *et al.*, 2007). There is increasing evidence that trichomes alter the feeding efficiency and oviposition preferences of various herbivores, including lepidopterans and coleopterans (e.g. Zvereva *et al*., 1998; Reymond *et al.*, 2004; Handley *et al.*, 2005; Sletvold *et al.*, 2010; Yamawo *et al.*, 2012). Plant trichomes are also known to show large phenotypic variation that is governed genetically (Ågren & Schemske, 1994; Kivimäki *et al.*, 2007; Bloomer *et al.*, 2012). Although trichomes exhibit quantitative phenotypic variation in their density or number (Ågren & Schemske, 1994), the presence/absence of trichomes (i.e. trichome-producing and trichomeless plants) is sometimes regulated by a few major genes (Kivimäki *et al.*, 2007; Kawagoe *et al.*, 2011; Bloomer *et al.*, 2012).

When anti-herbivore resistance traits are sufficiently varied within a plant community or population, the amount of herbivory on an individual plant depends not only on the plant's own phenotype but also on the neighbouring plant types (Atsatt & O'Dowd, 1976; reviewed by Agrawal *et al*., 2006; Barbosa *et al.*, 2009). This phenomenon is referred to as an associational effect and includes any consumer effect mediated by the composition of plant types within a neighbourhood on a focal individual plant (Underwood *et al.*, 2014). For example, palatable plants benefit from the presence of unpalatable plants via associational resistance if the latter repels herbivores away from the nearby palatable plants (Tahvanainen & Root, 1972). In contrast, unpalatable plants suffer from associational susceptibility when herbivores settle on palatable plants and then disperse onto unpalatable ones (White &

Whitham, 2000). Associational effects are predicted to influence plant diversity, because they may regulate population growth of multiple plant types in a density- or frequency-dependent manner (Hambäck *et al.*, 2014).

To date, associational effects have been mostly investigated as a type of interspecific interaction (e.g. Tahvanainen & Root, 1972; White & Whitham, 2000; Le Guigo *et al.*, 2012), but they can also be examined through the lens of intraspecific variation of anti-herbivore resistance traits (Wise *et al.*, 2009; Hambäck *et al.*, 2009). Several studies of cultivated plants have documented associational effects among resistant and susceptible genotypes within a plant species (Cantero & Sanford, 1984; Hambäck *et al.*, 2009). In these cases, a focal plant genotype may benefit via associational effects from the increasing proportion of another genotype in close vicinity. These interactions lead to frequency dependence in herbivory, which can promote the coexistence of different genotypes if rare genotypes are protected from herbivory (Wise *et al.*, 2009; Underwood *et al.*, 2014). Little is known about associational effects and their frequency dependence with respect to natural variation in anti-herbivore resistance traits within a plant species.

Arabidopsis halleri (L.) O'Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz [Brassicaceae/Cruciferae] has both trichome-producing (referred to hereafter as hairy) and trichomeless (glabrous) plants. The presence or absence of trichomes is associated with the allelic status of a candidate gene (Kawagoe *et al.*, 2011). Our previous study found that leaf damage on *A. halleri* depended on the relative frequency of neighbouring hairy and glabrous plants in a natural population where plants were heavily infested by an oligophagous leaf beetle, *Phaedon brassicae* Baly [Coleoptera: Chrysomelidae] (Sato *et al.*, 2014). In a choice experiment, we also revealed that when hairy plants were rare, they incurred less herbivory by *P. brassicae* than did glabrous plants,

whereas when hairy plants were common, they were not resistant to herbivory (Sato *et al.*, 2014). However, white butterflies, flea beetles, moths, and aphids can also be observed in natural populations of *A. halleri* (Y. Sato, personal observation), and the dominant herbivores vary even between closely located populations (Kawagoe & Kudoh, 2010). It is thus possible that patterns of associational effects depend on the herbivore species on the trichome dimorphism of *A. halleri*. Here, we examined whether such associational effects exist against herbivores other than the leaf beetle, *P. brassicae*.

In this study, two specific questions were addressed: (1) Does trichome production act as an anti-herbivore resistance trait, and if so, to which herbivores does it provides resistance? (2) Do any of the anti-herbivore effects of trichomes depend on the relative frequencies of hairy and glabrous plants? We performed both an experimental and an observational study to answer these questions. Firstly, we manipulated the relative frequency of hairy and glabrous plants in a common garden to test the resistance role of trichomes and their associational effects against herbivores. Secondly, we conducted a series of field surveys under different herbivore community structures to examine whether similar patterns of herbivory occurred in natural populations.

Materials and Methods

Study system

Arabidopsis halleri subsp. *gemmifera* is a perennial herb distributed across Japan and the Russian Far East (Al-Shehbaz & O'Kane, 2002). In the lowlands of western Japan, self-incompatible flowers start to bloom in late March or early April, and fruit set is ca. 80%

unless the flowers are consumed by herbivores (Kawagoe & Kudoh, 2010). After flowering, plants produce new rosettes on the main and axillary meristems of flowering stems, and these rosettes often establish as clonal offspring once they have rooted and attached themselves to the ground. Hairy and glabrous plants co-occur in a natural population in central Japan (Kawagoe *et al.*, 2011). The hairy and glabrous phenotypes in this population are associated with allelic variation in a candidate gene, *GLABROUS1* (*GL1*) but are not associated with other genes (Kawagoe *et al.*, 2011; see Grebe, 2012 for a review of *GL1* in *A. thaliana*). Hairy plants develop trichomes on the surfaces of their leaves and stems, but not on the flowers and fruits, whereas glabrous plants have no trichomes except along the leaf margin. The two morphs have no other apparent morphological differences. Hairy plants produced fewer fruits than glabrous plants under weak herbivory (Kawagoe *et al.*, 2011), indicating a trade-off with trichome production. Glucosinolate profiles were not associated with either the hairy or the glabrous phenotype in our seed source population, as described below (Sato *et al.*, 2014).

Common garden experiment

Mature fruits of *A. halleri* were collected from a natural population located in Taka-cho, Hyogo Prefecture, central Japan (35°06′N, 134°56′E, ca. 200 m in altitude, Sato *et al.*, 2014). The fruits were harvested from 14 maternal plants (including seven hairy and seven glabrous plants) in early July 2011; the maternal plants were spaced at least 5 m apart to minimize the likelihood of multiple samples being taken from a single clone. These fruits provided the seeds from which the plants in our experiment were grown.

For the common garden experiment, we initially prepared ca. 200 plants in an indoor space that contained no herbivores. Fifty seeds from each maternal family were sown

on a petri dish (diameter 9 cm, depth 1.5 cm) filled with moistened quartz sand on 15 August 2011 and were allowed to germinate at room temperature. Three seedlings per family were transplanted to a plastic pot (diameter 10.5 cm, depth 9 cm) filled with mixed soil (pumice:leaf mold:peat moss = 1:1:1) on 25 November 2011. All seedlings had two to four leaves when they were transplanted. To prevent herbivory, these plants were placed inside transparent plastic cases (75 cm × 45 cm with a depth of 18 cm, 28 pots per case). The plants grew for four months under natural sunlight (10–12 h in day length) with average daily temperatures of 10–30 °C. The location of the cases was rotated every month. All plants were transplanted individually into plastic pots about a month before the start of the experiment to avoid competition within the cases. The liquid fertiliser Hyponex (Hyponex, N:P:K = 6:10:5, Hyponex Japan, Osaka, Japan) was diluted 1000-fold and supplied monthly until the beginning of the experiment. Photosynthetically active radiation was 800–1100 and 100–300 μmol/m^2/s on a sunny and cloudy day, respectively. The red:far-red ratio ranged from 1.0 to 1.3 (LI-190 Quantum Sensor, LI-COR, Lincoln, NE, USA).

The potted plants were transferred to the experimental garden of the Center for Ecological Research, Kyoto University (34°58′N, 135°57′E, ca. 100 m in altitude) on 28 March 2012. All cultivated plants were sorted in ascending order of leaf length (the length of the longest radical leaf, which ranged from 33 to 71 mm), and the largest 150 plants from the 14 maternal families were used in the experiments, in order to ensure that similarly sized plants were used. We controlled for plant size to avoid confounding effects on herbivore abundance per plant.

Twenty-five individuals were arranged squarely in each of six 1 × 1 m plots filled with fine gravel. We established two treatments for the frequency of hairy and glabrous plants within a plot (Fig. 1); three plots consisted of 21 hairy and four glabrous plants

(referred to as hairy-abundant plots), and the remaining three consisted of four hairy and 21 glabrous plants (referred to as glabrous-abundant plots). The plots were spaced 1.7 m apart. The hairy or glabrous plants of similar size (< 11 mm difference in the length of the longest radical leaf within a single plot) were placed in the centre of the plot, while plants of random size occupied the positions at the edge (Fig. 1).

Herbivore abundance and plant performance were monitored weekly on sunny days from April to June 2012 (2, 9, 17, 25, and 30 April; 7, 14, 21, and 28 May; 4, 11, 18, and 25 June). The mustard aphid *Lipaphis erysimi* Kaltenbach [Hemiptera: Aphididae] and green peach aphid *Myzus persicae* Sulzer [Hemiptera: Aphididae] occurred on *A. halleri* at our experimental site (see Supporting information, Fig. S1). The small cabbage white butterfly *Pieris rapae* L. [Lepidoptera: Pieridae], cabbage sawfly *Athalia infumata* Marlatt [Hymenoptera: Tenthredinidae], and flea beetle *Phyllotreta striolata* Fabricius [Coleoptera: Chrysomelidae] also occurred, mainly after the flowering season (Table 1; Fig. S1). Wingless and winged aphids were counted separately, as the former represented a growing colony while the latter was likely to be associated with dispersal events. We counted *L. erysimi* and *M. persicae* as a single group because the two species were difficult to distinguish accurately in the field due to their similar colours and morphology at the nymph stage. In addition, we recorded the number of mummies (i.e. aphids fed on by parasitoid wasps) after the peak abundance of wingless aphids (14 May 2012). The numbers of intact and damaged leaves were recorded separately in order to distinctly evaluate plant growth and herbivory damage. The numbers of flowers and mature fruits were counted weekly for each plant. We counted flowers as the number of pedicels, including both fruited and non-fruited flowers, to estimate the flower production of each plant throughout the season. The seed production of each plant could not be evaluated because mature seeds spontaneously dropped; therefore, we used the number of mature fruits to estimate seed production. A mature fruit contained 8.3 ± 2.2 seeds

188 under our experimental conditions (mean ± SD, *n* = 19 fruits). To avoid edge effects in the
189 plots, we analysed the nine plants in the centre of each plot (Fig. 1). Three edge plants died
190 from unknown causes (not related to herbivory levels). All measurements were carried out
191 between 8:00 and 17:00.

192

193 *Field survey*

194 We conducted field surveys in two natural populations of *A. halleri*; Ojigahata, Shiga
195 (35°12′N, 136°23′E, ca. 300 m in altitude) and Minoh, Osaka, Japan (34°50′N, 135°28′E, ca.
196 50 m in altitude). The two sites were selected because the green-veined white butterflies,
197 *Pieris napi* L. [Lepidoptera: Pieridae], were predominantly observed at one site (Ojigahata)
198 while the flea beetles *Phyllotreta striolata* were predominantly observed at the other site
199 (Minoh). Both sites were located in gravel areas near roads that were covered with sparse
200 vegetation. The population size and proportion of hairy plants were similar between the two
201 sites (no. of plants = 500–1000, percentage of hairy plants = 40–60%). We observed other
202 cruciferous plants (*Cardamine hirsuta*, *C. impatiens*, *C. leucantha*, and *C. scutata*) at these
203 sites, but they occurred only rarely.

204 Field surveys were conducted twice a year in spring (late May or early June) and
205 autumn (late September or early October), as *P. napi* shows bivoltinism (Fukuda *et al.*, 1984).
206 For each population and census, we recorded the trichome phenotype (hairy or glabrous) and
207 the proportion of leaf area lost to herbivory for all individual plants in randomly chosen
208 circular patches (1 m in diameter). Our preliminary survey confirmed that the number of
209 plants within each circular patch began to plateau as the patch size increased. At the
210 Ojigahata site, 2.92 ± 0.25, 5.52 ± 0.70, and 7.40 ± 1.02 plants occurred within 0.5-, 1.0-, and
211 3.0-m–diameter patches, respectively (mean ± SE, *n* = 25 patches examined). At the Minoh

site, 3.71 ± 0.29, 7.68 ± 0.61, and 10.68 ± 0.95 plants occurred within 0.5-, 1.0-, and 3.0-m–diameter patches, respectively (mean ± SE, n = 28 patches). Therefore, we focused on the local interaction in 1-m–diameter patches. We examined 70–90 patches, including 150–500 hairy and 200–550 glabrous plants, at the Ojigahata site; and we examined 65–85 patches, including 180–350 hairy and 290–380 glabrous plants, at the Minoh site (see also Table 2 and 3 for detailed survey dates and sample sizes). The proportion of leaf area lost to herbivory (referred to hereafter as the leaf damage) was evaluated visually and recorded as one of 11 successive values: 0 (no damage), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 (complete leaf loss). In addition, to evaluate to what extent our method of quantifying the leaf damage reflected the intensity of herbivory, we also recorded the number of intact and damaged leaves of 19 plants at the Ojigahata site. This additional measurement confirmed that the leaf damage estimated by our method was highly correlated with the total proportion of leaves damaged (Pearson's product moment correlation; both variables were arcsine-transformed, r = 0.92, t_{17} = 9.4, P < 0.0001). We also identified and counted herbivorous insects within the study area during the survey. All observations and surveys were carried out for 4–6 hours between 9:00 and 16:00.

Statistical analysis

We used the data collected in our experiment to analyse the effects of the trichome phenotype (hairy or glabrous phenotype for each plant) and frequency condition (hairy-abundant or glabrous-abundant for each plot) as fixed effects explaining the number of herbivores, flowers, or leaves per plant (response variables). The interaction term of the trichome phenotype × frequency condition was incorporated as a fixed effect to determine whether the main effects of the trichome phenotype on the herbivore abundance or plant performance

236 depended on the frequency condition (namely, associational effects). To analyse the count
237 response, we used generalised linear mixed models (GLMMs: Bolker *et al.*, 2009) with a
238 Poisson error structure and a log link function. The significance levels of the main effects
239 were first analysed using likelihood ratio tests. Then, the trichome-by-frequency interaction
240 was analysed to test associational effects. The cumulative number of each herbivore per plant
241 was analysed as the response variable to reflect both herbivory intensity and duration for
242 plants (i.e. herbivory load: Ruppel, 1983; Fournier *et al.*, 2005). As an exception, the number
243 of mummified aphids was analysed using the abundance that was recorded on 28 May
244 because the mummified aphids were attached to plant surfaces and reflected the accumulated
245 number by themselves.

246 Aphids were observed on leaves throughout the study, whereas white butterflies,
247 cabbage sawflies, and flea beetles were observed only during June (Fig. S1). Thus, to exclude
248 the direct confounding interactions of the late-coming herbivores, we separately analysed the
249 cumulative number of wingless and winged aphids counted per plant before 28 May. To
250 examine plant fecundity and growth corresponding to this period, we analysed the data
251 collected until the end of May regarding the number of flowers and intact leaves. The number
252 of mature fruits was also analysed; however, the result is not presented in the main text
253 because it followed a similar pattern to that of the number of flowers during the early period
254 of the experiment (Fig. S2) and no significant effects were detected regarding the number of
255 fruits (see Table 1 and Results section below). A small number of leaves (median = 0 leaves)
256 were damaged in the period before 28 May 2012 (Fig. S2); therefore, the data for this
257 variable during the early period were not analysed.

258 For the late-coming herbivores (white butterflies, sawflies, and flea beetles), we
259 analysed the cumulative number of eggs and larvae of *P. rapae*, larvae of *A. infumata*, and

adults of *P. striolata*, in which the cumulative number of each herbivore was calculated throughout the study. Then, to examine the effects of the late-coming herbivores on plant growth, we analysed the number of damaged leaves at the end of the study (25 June 2012). Many leaves were damaged by the end of the experiment (Fig. S2); thus, their number was sufficient to estimate the degree of herbivory on the two types of plants. The number of mature fruits during the late season was not analysed because the impact of these late-coming insects was so severe that a large proportion of the fruits was lost to herbivory.

In some of the GLMM analyses, we included an offset term of $\log_e (x + 1)$ to examine the rates of the count response (Nelder & Frome, 1984). This method allows us to handle the effects of sampling effort, or total counts, as a covariate without estimating a slope coefficient in the analysis of the count response. For the analyses of winged and mummified aphids, we included the number of wingless aphids as an offset term to consider the density-dependent dispersal or parasitism on living aphids. The number of flowers was included as an offset term to analyse fruit set (that is, the proportion of mature fruits in the total number of flowers produced) for the number of mature fruits. To analyse the number of damaged leaves, we included the number of intact leaves as an offset term to evaluate the per capita leaf damage.

We incorporated two random effects into all of the GLMMs above. Firstly, to reflect the variance of herbivore abundance and plant performance among the plots in the analyses, we included the plot ID as a random effect. Secondly, because we designed the experiment to control for plant size, the number of individual plants differed between maternal families (mean ± SD = 4.2 ± 2.8, n = 14 maternal plants). Thus, the seed plant ID was incorporated as another random effect to consider the potential variation in other plant traits related to the maternal families.

We used the field survey data to analyse the effects of the trichome phenotype, the proportion of glabrous plants in a patch (which represents the relative frequency of the two plant types), and the total number of plants (which represents the density of *A. halleri* plants) as fixed effects explaining the leaf damage. Leaf damage was analysed using linear mixed models, in which the response variable was arcsine-square root transformed to improve the normality of residuals. To test whether the effect of the trichome phenotype on leaf damage depended on the proportion of glabrous plants (i.e. associational effects), we also analysed the interaction between the trichome phenotype and the proportion of glabrous plants in a patch as another fixed effect. The patch ID was incorporated as a random effect so that multiple individuals in a patch were not treated as independent samples. We used likelihood ratio tests to analyse the significance of each fixed effect, in which the main effects were first tested and then the interaction term was examined. Furthermore, in the linear mixed models, we estimated coefficients of the terms 'the proportion of glabrous plants in a patch' and 'the total number of *A. halleri* in a patch' to examine the sign and magnitude of the effects of the frequency of hairy and glabrous plants and their density on leaf damage. When the coefficients significantly deviated from zero, we added trend lines to figures using single regression of the leaf damage on the proportion of glabrous plants in a patch. These analyses were separately performed for each survey at the Ojigahata and Minoh sites.

All statistical analyses were performed using R version 2.15.0 (R Development Core Team, 2012). We used the glmer and lmer functions (in the lme4 package: Bates *et al.*, 2012) for GLMMs and linear mixed model analyses. We chose the maximum likelihood method for the lmer function and the Laplace approximation for the glmer function to estimate likelihoods and coefficients.

Results

Common garden experiment

Aphids emerged in late April, followed by *P. rapae*, *A. infumata*, and *P. striolata* (Fig. S1). We also observed a few *Thrips palmi* Karny [Thysanoptera: Thripidae] and *Plutella xylostella* L. [Lepidoptera: Yponomeutidae] on *A. halleri* during June, but their abundance was not recorded because they were rare. Three of the 240 *P. rapae* larvae counted in this study were attacked by parasitoid wasps. Although we counted aphids as a single herbivore group, detailed identification under a binocular microscope on 30 April 2012 revealed that 67 specimen samples contained 24 individuals of *L. erysimi* and 43 individuals of *M. persicae*.

During the early period, the number of wingless aphids did not differ between hairy and glabrous plants (Fig. 2a: Table 1), indicating little resistance role of the trichome production against the aphids. Wingless aphids occurred slightly more often on hairy plants under the glabrous-abundant condition (the trichome × frequency interaction, $P < 0.05$: Table 1; Fig. 2a). The number of winged aphids was not significantly affected by the trichome phenotype (Table 1; Fig. 2b). Like the wingless aphids, mummified aphids occurred more often on hairy plants under the glabrous-abundant condition (Table 1; Fig. 2c). During this early period, glabrous plants showed a higher flower production than hairy plants (the main effect of the trichome phenotype, $P < 0.05$: Table1; Fig. 2d). The fruit production was not significantly affected by the trichome phenotype, frequency condition, and their interaction (Table 1; Fig. S2). The number of intact leaves did not differ between hairy and glabrous plants (the main effect of the trichome phenotype, $P = 0.68$: Table 1), but glabrous plants had a larger number of intact leaves than hairy ones under the glabrous-abundant condition (the trichome × frequency interaction, $P < 0.05$: Table 1; Fig. 2e).

During the later period of the experiment, *P. rapae* eggs, *P. rapae* larvae, and *A. infumata* larvae tended to occur less often on hairy plants within the frequency condition (the main effect of the trichome phenotype, $P < 0.05$, $P = 0.07$, and $P = 0.42$ for *Pi. rapae* eggs, *P. rapae* larvae, and *A. infumata* larvae, respectively: Table 1; Figs. 2f, g, h), providing evidence for a resistance role of the trichome production against these herbivores. The number of *P. rapae* and *A. infumata*, was not interactively affected by the trichome phenotype and frequency condition (Table 1), indicating no associational effects. Adults of the flea beetle, *P. striolata*, occurred slightly more frequently on hairy plants (the main effect of the trichome phenotype, $P = 0.07$: Fig. 2i), but there was no evidence for associational effects (i.e. no significant trichome × frequency interaction: Table 1). During this later period, hairy plants tended to have fewer damaged leaves than did glabrous plants (Fig. 2j), although the main effect of the trichome phenotype was not significant (Table 1).

Field survey

The green-veined white butterfly, *Pieris napi*, was a major insect herbivore in the Ojigahata site, whereas the flea beetle, *Phyllotreta striolata*, was dominant in the Minoh site (Table 2a). On 15 September 2012 at the Ojigahata site, we found one egg on a hairy plant and eight eggs on glabrous plants. On 27 May 2013 at the Ojigahata site, two eggs were found on glabrous plants and we observed a larva of *P. napi*. Larvae of *A. infumata* were found occasionally at the Ojigahata and Minoh site (Table 2a). We did not observe any parasitoids attacking herbivorous insects in the two populations.

There was less leaf damage on hairy plants than on glabrous plants over the course of two years at the Ojigahata site (the main effect of the trichome phenotype, $P < 0.05$ over the four surveys: Table 2; Fig. 3a). On the other hand, no consistently significant patterns

were observed between the leaf damage on hairy and glabrous plants over the course of two years at the Minoh site (Table 2; Fig. 3b). In these two populations, we rarely found significant interactions (2 out of 16 cases) between the trichome phenotype and the proportion of glabrous plants in a patch (except for 15 September 2012 and 26 May 2013 at the Ojigahata site, $P < 0.05$: Table 2), indicating little associational effects on the leaf damage. The proportion of glabrous plants in a patch was not significantly related to leaf damage in 13 out of 16 cases (except for one case at the Ojigahata site and two cases at the Minoh site: Table 3; Fig. 4). The total number of *A. halleri* in a patch showed few or negative effects from leaf damage; six out of 16 cases were significantly negative (two cases at the Ojigahata site and four cases at the Minoh site: Table 3).

Discussion

In this study, we were unable to detect any conclusive evidence of associational effects; however, we did find evidence to suggest a role for trichomes as an anti-herbivore resistance trait against certain herbivores. In the common garden experiment, we observed fewer eggs and larvae of the small cabbage white, *Pieris rapae*, on hairy plants than on glabrous plants. We also found that larvae of the cabbage sawfly, *Athalia infumata*, tended to occur less often on hairy plants than on glabrous plants. During the later period when these two species of leaf chewers were abundant, hairy plants tended to suffer less leaf damage than glabrous plants. In contrast, we did not observe a reduced abundance of aphids (*Myzus persicae* and *Lipaphis erysimi*) and flea beetles (*Phyllotreta striolata*) on hairy plants in the common garden. In the field surveys, hairy plants incurred slightly less herbivory than glabrous plants in a natural population where the green-veined white butterfly, *Pieris napi*, was dominant. In contrast, no consistent differences in leaf damage were detected between

hairy and glabrous plants in the other population where *P. napi* was rarely observed and where the flea beetle was dominant.

Anti-herbivore resistance functions of trichomes have been reported for interactions between other *Arabidopsis* trichomes and leaf-chewing insects. Leaf trichomes are known to prevent damage by the diamondback moth *Plutella xylostella* in *A. lyrata* (Sletvold *et al.*, 2010) and to reduce the performance of *P. rapae* larvae in *A. thaliana* (Reymond *et al.*, 2004; reviewed by van Poecke, 2007). In addition, in *A. halleri*, our previous study revealed that trichome production influenced the feeding preferences of adults and reduced the performance of larvae of the leaf beetle *Phaedon brassicae* (Sato *et al.*, 2014). Given the common feeding habit among the white butterflies and sawflies (which tended to occur less often on hairy plants in the common garden) in addition to the leaf beetles, our findings suggest a role for trichomes in resistance against leaf chewers (Table 4). In *A. thaliana* and *A. lyrata*, trichomes are also known to repel oviposition by *P. xylostella* (Handley *et al.*, 2005; Sletvold *et al.*, 2010). The result that fewer eggs of *P. rapae* were observed on hairy plants in our common garden experiment may support such negative effects of trichomes on the oviposition preference of lepidopterans.

Plant trichomes are not always effective as a mechanism of herbivore resistance. Their effects depend on herbivore feeding habits (Andres & Conner, 2003), herbivore life stage (Yamawo *et al.*, 2012), and plant ontogeny (Puentes & Ågren, 2013). For instance, in a community-level study of manzanita (*Arctostaphylos*) species, Andres and Conner (2003) suggested that trichomes were ineffective as a resistance trait against small or sedentary herbivores because such herbivores have less contact with plant hairs during their life cycles. In our study, aphids infrequently contacted *A. halleri* trichomes because the flowering buds of *A. halleri* did not produce trichomes, and many of the aphids were observed on the tops of

flowering stems (Y. Sato, personal observation). In addition, young leaves of hairy plants produce ca. 60 trichomes/cm^2, but the trichome density becomes low (ca. 10 trichomes/cm^2) in mature leaves (Y. Sato, unpublished data). Thus, flea beetle (*Phyllotreta striolata*) adults, which have a body size of a few millimetres, contacted few hairs at such a sparse trichome density. Furthermore, adult flea beetles can move from one leaf to another by jumping, so trichomes are unlikely to interfere with their mobility (Table 4). The potential role of trichomes in resistance should be tested with regards to plant ontogeny in order to fully understand factors determining the effectiveness of trichomes against herbivores.

Regarding associational effects in morphological traits, Wise *et al.* (2009) reported that resistance to gall flies did not depend on the frequency of the erect-stemmed and candy-cane phenotypes of *Solidago altissima* in 1.5-m^3 cages. Here, we also found limited evidence of associational effects between white butterflies and the trichome phenotypes of *A. halleri*. Given that adult white butterflies can move among plant patches by flying, we speculate that associational effects are unlikely to cause resistance to oviposition by the butterflies at our 1-m patch scale (Table 4). Although this study did not detect associational effects, our previous study found that the magnitude of herbivory on hairy plants depended on the proportion of glabrous plants within 1-m diameter patches in another population where the flightless leaf beetle *Phaedon brassicae* was the predominant herbivore on *A. halleri* (Table 4; Sato *et al.*, 2014). Taken together, our data suggest that herbivore mobility affects the occurrence of associational effects between herbivory and trichome phenotype of *A. halleri* (Table 4).

As for another ecological function, plant trichomes are known to interfere with the foraging behaviour of predators (e.g. Kauffman & Kennedy, 1989; reviewed by Dalin *et al.*, 2008). We observed a parasitoid, *Aphidius* sp., in the experimental plots, but the number of parasitized aphids was not fewer on hairy plants. Thus this did not support such interfering

effects of trichomes on foraging of predators. However, it is still possible that some confounding interactions among herbivores have hindered associational effects. Firstly, size reduction of glabrous plants by two leaf chewers (*Pieris rapae* and *Athalia infumata*) may account for the slightly higher number of *Phyllotreta striolata* on hairy plants in the common garden. Secondly, the aphid colony contained a specialist, *Lipaphis erysimi*, and a generalist, *Myzus persicae* (Rout & Senapati, 1968; Le Guigo *et al.*, 2012); therefore, it is possible that anti-herbivore resistance or its associational effects could have been detected if the two species had been distinctly evaluated.

Although the result that abundance of *Pieris rapae* larvae were reduced on hairy plants are congruent with the pattern of leaf damage in a natural population where *Pieris napi* were dominant, one caveat is that *P. rapae* does not utilize *A. halleri* in natural populations (Ohsaki & Sato, 1999). Ohsaki and Sato (1999) showed that larvae of *P. rapae* were able to grow on *A. halleri*, but adults did not oviposit on *A. halleri* when they had access to other cruciferous plants, such as *Brassica* and *Raphanus* species. Thus, our results of the common garden experiment should be interpreted carefully about to what extent they are comparable to patterns of leaf damage in a population where a congener, *P. napi*, is the dominant herbivore.

In conclusion, although this study does not provide any conclusive evidence of associational effects of anti-herbivore resistance, we did find that trichomes can act as a resistance trait against certain herbivores in *A. halleri*. However, we should note that conditions under which associational effects can be detected sometimes depend on spatial scales (Janz *et al.*, 2005; Hambäck *et al.*, 2009). For example, Janz *et al.* (2005) illustrated that the oviposition preference of a polyphagous butterfly, *Polygonia c-album*, did not depend on the frequency of *Urtica dioica* and *Salix caprea* within a patch, but such frequency-

dependent oviposition was found among plant patches. In our study, it remains possible that associational effects against flying herbivores (e.g. adults of *Pieris* species) can be detected at a spatial scale greater than 1 m^2. Thus, in future studies, it would be valuable to examine the effects of spatial scales in order to understand when associational effects occur in anti-herbivore resistance.

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584 **Table 1.** Effects of the trichome phenotype and frequency condition on the cumulative number of herbivores and plant performance (the
585 number of flowers or leaves) in the experiment. Likelihood ratio tests were performed using generalised linear models (GLMMs). Early-
586 season herbivores (aphids) and late-season herbivores (*Pieris rapae*, *Athalia infumata*, and *Phyllotreta striolata*) were separately analysed.
587 Plant performance was also separately analysed according to the analyses of herbivore abundance. Main effects were tested first and then
588 an interaction term was analysed. The bold and underlined values indicate < 5% significance and marginal (5%–10%) significance,
589 respectively.

Fixed effects	#Wingless aphids			#Winged aphids*			#Mummified aphids*			#Flowers			#Matured fruits**			#Intact leaves		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	1	0.7	0.39	1	0.4	0.52	1	3.6	0.06	**1**	**3.9**	**0.05**	1	0.2	0.65	1	0.17	0.68
Frequency	1	0.7	0.39	1	2.5	0.12	1	0.001	0.98	**1**	**4.7**	**0.03**	1	0.01	0.93	1	2.1	0.15
Tri. × Freq.	**1**	**5.7**	**0.02**	1	2.8	0.10	1	3.7	0.05	1	3.5	0.06	1	0.04	0.84	**1**	**10.7**	**0.001**
Residuals	48	860	---	48	73.6	---	48	80.2	---	48	84.9	---	48	32.4	---	48	242.4	---

*No. of wingless aphids was included as an offset term; **No. of flowers was included as an offset term.

Fixed effects	#*P. rapae* eggs			#*P. rapae* larvae			#*A. infumata* larvae			#*P. striolata* adults			#Damaged leaves***		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	**1**	**5.4**	**0.02**	1	3.3	0.07	1	0.6	0.42	1	3.2	0.07	1	2.3	0.13
Frequency	1	3.0	0.09	1	0.03	0.87	1	0.2	0.62	1	0.2	0.65	1	0.03	0.87
Tri. × Freq.	1	0.01	0.93	1	0.5	0.47	1	0.002	0.97	1	0.5	0.50	**1**	**138.7**	**< 0.001**
Residuals	48	54.8	---	48	98.4	---	48	67.8	---	48	56.1	---	48	791.4	---

***No. of intact leaves was included as an offset term.

590 **Table 2.** Insect herbivores counted in the study sites (a) and effects of the trichome phenotype, proportion of glabrous plants in a patch, and
591 total number of *Arabidopsis halleri* plants in a patch on leaf damage (arcsine-transformed proportion of the leaf area loss) (b) at the
592 Ojigahata site and (c) at the Minoh site. Likelihood ratio tests were performed using linear mixed models. Main effects were tested first and
593 then an interaction term was analysed. The bold and underlined values indicate < 5% significance and marginal (5%–10%) significance,
594 respectively. The patch ID was incorporated as a random effect in these analyses.

(a) Insect herbivores

Species	2011 Autumn		2012 Spring		2012 Autumn		2013 Spring	
	Ojigahata	Minoh	Ojigahata	Minoh	Ojigahata	Minoh	Ojigahata	Minoh
Pieris napi adults*	8	0	8	0	22(9)	0	8(3)	0
Phyllotreta striolata adults	0	0	15	3	2	0	33	0
Athalia infumata larvae	0	0	1	7	0	0	0	0

*No. of eggs and larvae are shown within parentheses.

(b) Ojigahata

Fixed effects	23 September 2011			27 May 2012			15 September 2012			26 May 2013		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	**1**	**20.8**	**< 0.001**	**1**	**7.3**	**< 0.01**	**1**	**4.1**	**0.04**	**1**	**36.1**	**< 0.001**
Proportion of glabrous plants	**1**	**5.9**	**0.02**	1	0.4	0.56	1	0.1	0.76	1	0.9	0.34
Total number of plants	1	0.48	0.49	1	0.05	0.83	**1**	**5.0**	**0.03**	1	2.1	0.15
Tri. × Prop. of glabrous plants	1	0.50	0.48	1	0.01	0.91	**1**	**4.8**	**0.03**	**1**	**4.5**	**0.03**
Residuals	1016	-844.4	---	505	-437.3	---	383	-310.9	---	441	-393.1	---

(c) Minoh

Fixed effects	1 October 2011			3 June 2012			22 September 2012			1 June 2013		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*

Trichome	1	3.7	0.06	1	0.01	0.93	**1**	**37.2**	**< 0.001**	**1**	**13.6**	**< 0.001**
Proportion of glabrous plants	1	1.5	0.22	1	0.01	0.93	1	0.8	0.37	**1**	**5.0**	**0.025**
Total number of plants	1	1.0	0.31	1	3.8	0.05	**1**	**11.1**	**0.001**	**1**	**7.3**	**< 0.01**
Tri. × Prop. of glabrous plants	1	1.3	0.26	1	2.2	0.13	1	0.3	0.57	1	3.6	0.06
Residuals	691	-687	---	471	-371.9	---	561	-553.1	---	571	-409.6	---

605 **Table 3.** Effects of the proportion of glabrous plants in a patch and the total number of *Arabidopsis halleri* plants in a patch on leaf damage
606 (arcsine-transformed proportion of the leaf area loss) (a) at the Ojigahata site and (b) at the Minoh site. Standardized coefficients and their
607 standard error (SE) in linear mixed models explaining the leaf damage are shown for the two factors, in which bold values indicate < 5%
608 significant deviation of coefficients from zero (Wald tests). The patch ID was incorporated as a random effect in these analyses. The
609 number of plant patches examined is shown within the parentheses beside the survey dates, and the number of hairy or glabrous plants
610 examined is presented within the parentheses below each survey date. The observational evidence of herbivore fauna was described in
611 Table 2.

(a) Ojigahata

Fixed effects	23 September 2011 (80)		27 May 2012 (72)		15 September 2012 (90)		26 May 2013 (70)	
	Hairy (498)	Glabrous (523)	Hairy (180)	Glabrous (330)	Hairy (178)	Glabrous (210)	Hairy (156)	Glabrous (290)
Proportion of glabrous plants	-0.07 ± 0.06	**-0.12 ± 0.06**	0.08 ± 0.08	0.01 ± 0.06	-0.12 ± 0.07	0.10 ± 0.09	0.09 ± 0.08	-0.08 ± 0.07
Total number of plants	-0.02 ± 0.08	-0.02 ± 0.07	0.07 ± 0.08	0.002 ± 0.066	**-0.16 ± 0.07**	-0.15 ± 0.10	**-0.23 ± 0.08**	-0.02 ± 0.08

(b) Minoh

Fixed effects	1 October 2011 (67)		3 June 2012 (66)		22 September 2012 (85)		1 June 2013 (70)	
	Hairy (340)	Glabrous (356)	Hairy (186)	Glabrous (290)	Hairy (249)	Glabrous (317)	Hairy (197)	Glabrous (379)
Proportion of glabrous plants	-0.04 ± 0.09	**-0.23 ± 0.07**	-0.07 ± 0.10	0.07 ± 0.08	-0.03 ± 0.09	-0.08 ± 0.07	**0.35 ± 0.08**	0.03 ± 0.07
Total number of plants	-0.06 ± 0.11	-0.12 ± 0.09	**-0.19 ± 0.10**	-0.11 ± 0.08	**-0.31 ± 0.13**	**-0.26 ± 0.09**	-0.08 ± 0.09	**-0.27 ± 0.10**

612 **Table 4.** Patterns of anti-herbivore resistance and associational effects between different herbivores and hairy and glabrous plants of
613 *Arabidopsis halleri*. Feeding habit, dispersal mode, and body length of these herbivores (less than the maximum size throughout their life
614 cycle) are also listed. Circles and cross marks indicate that associational effects were supported and not supported, respectively.
615 Associational effects cannot be discussed regarding herbivores in which trichomes do not serve as a resistance trait (indicated by 'NA').

Herbivore	Species	Feeding habit	Dispersal	Body length	Resistance effect of trichomes	Associational effects between trichome phenotypes	References
Leaf beetle	*Phaedon brassicae*	Chewer	Walking	< 1.0 cm	○	○	Sato *et al.*, 2014
White butterflies	*Pieris rapae*, *Pieris napi*	Chewer	Flying (adult)	< 4.0 cm	○	×	This study
Cabbage sawfly	*Athalia infumata*	Chewer	Flying (adult)	< 2.0 cm	○	×	This study
Flea beetle	*Phyllotreta striolata*	Chewer	Jumping	< 0.3 cm	×	NA	This study
Aphids	*Myzus persicae*, *Lipaphis erysimi*	Sucker	Walking + Flying	< 0.3 cm	×	NA	This study

616 **Figure legends**

617

618 **Figure 1.** A schematic diagram showing the experimental design of the experiment in which
619 the relative frequency of hairy and glabrous plants was manipulated. The hairy-abundant (H
620 > G) plot and glabrous-abundant plot (H < G) consisted of 84% and 16% hairy plants,
621 respectively. Twenty-five plants were planted in a grid, and three replications were set for
622 each plot type. In the left two panels, a filled or open square indicates a hairy (H) or glabrous
623 (G) plant, respectively. Analysed plants (nine individuals in the centre of each plot) were
624 sorted by rosette size, and the number after the 'H' or 'G' indicates the ascending order of
625 sorting. The rest of the hairy or glabrous plants were placed on the edge of the plots with
626 randomization of plant size. Plants were maintained at intervals of 20 cm.

627 **Figure 2.** The cumulative number (mean ± SE) of herbivorous insects (a–c, f–i) and the
628 number of flowers (d) and leaves (e, j) of the hairy (H; coloured) and glabrous (G; white)
629 plants in the hairy-abundant (H > G) and glabrous-abundant (H < G) plots. Data are
630 separately analysed for the early- and late-season surveys to distinguish the effects of
631 herbivory between the two periods (see also text and Figs. S1, S2).

632 **Figure 3.** Leaf damage (proportion of the leaf area loss: mean ± SE) of hairy (H; coloured)
633 and glabrous (G; white) plants (a) at the Ojigahata site and (b) at the Minoh site. Data are not
634 transformed in the figure. Detailed survey dates and observational evidence of herbivore
635 fauna are provided in Table 2.

636 **Figure 4.** Leaf damage (proportion of the leaf area loss) on hairy (coloured circles) or
637 glabrous (white circles) plants in relation to the increasing proportion of neighbouring
638 glabrous plants (a) at the Ojigahata site and (b) at the Minoh site. Circles and vertical bars
639 respectively indicate the mean and SE of leaf damage on individual plants within a 1-m
640 diameter patch. Note that many points do not have error bars because all plants within a patch
641 received the same damage score. Trend lines (dashed lines) were added on the basis of single
642 regressions when the slope coefficients were significant. Darker circles indicate a larger
643 number of plants in a patch. Data are not transformed in the figure. Detailed survey dates are
644 described in Table 2 and 3.

645

646 **Figure legends for supporting information**

647

648

649 **Figure S1.** Temporal patterns in the number of each herbivore on the hairy (coloured) and
650 glabrous (white) plants in the hairy-abundant (left) and glabrous-abundant (right) plots
651 throughout the study. Mean ± SE values are shown.

652

653

654 **Figure S2.** Temporal patterns in the performance of hairy (coloured) and glabrous (white)
655 plants in the hairy-abundant (left) and glabrous-abundant (right) plots throughout the study.
656 Mean ± SE values are shown. Flowers were counted as the total number of pedicels per plant,
657 including both fruited and non-fruited flowers.

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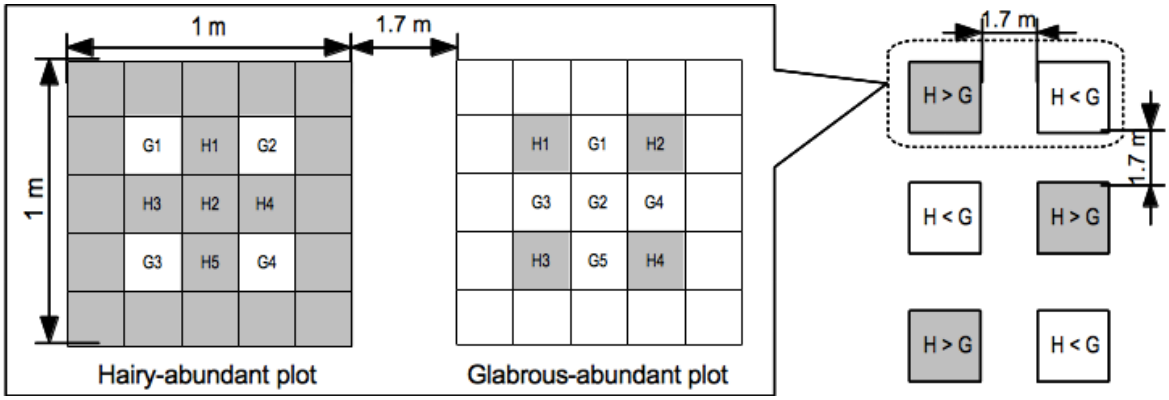
667 **Figure 1**



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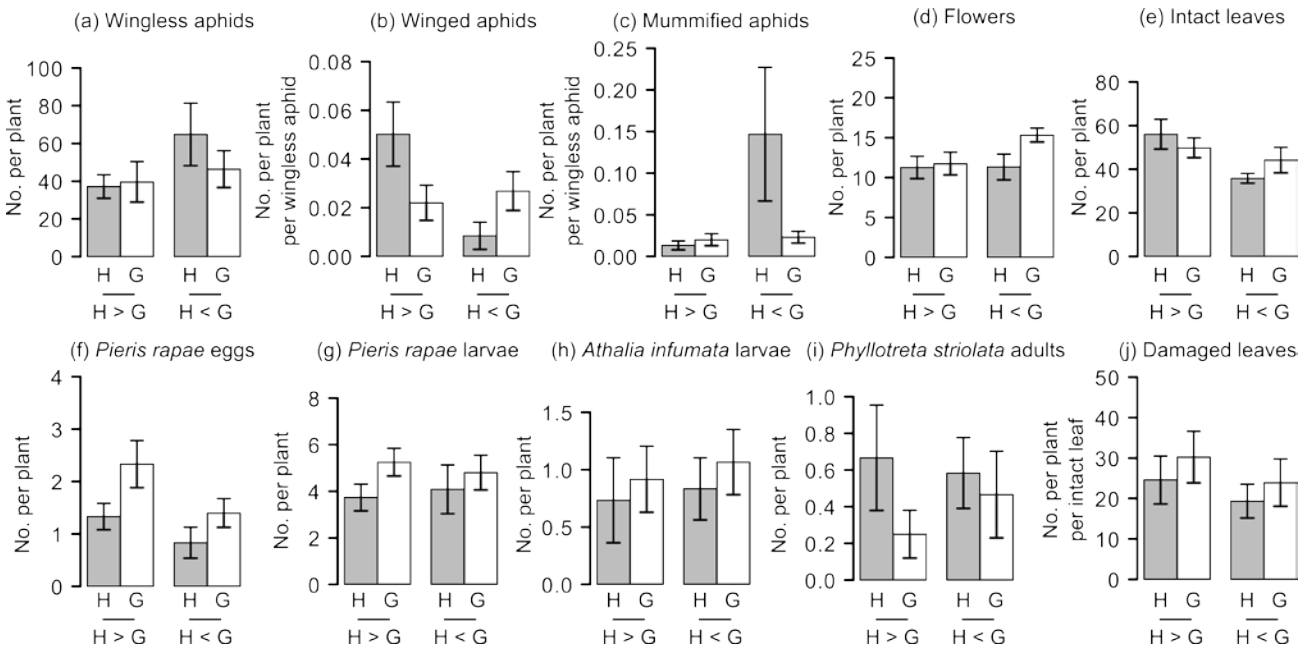
682 **Figure 2**



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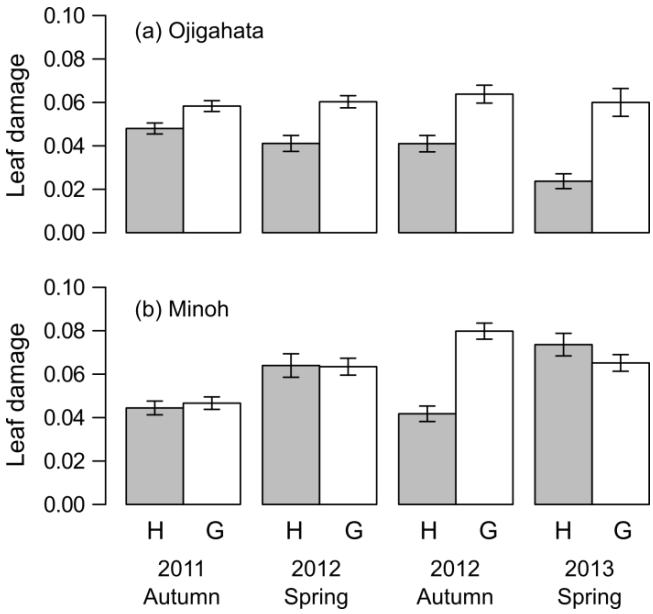
694 **Figure 3**



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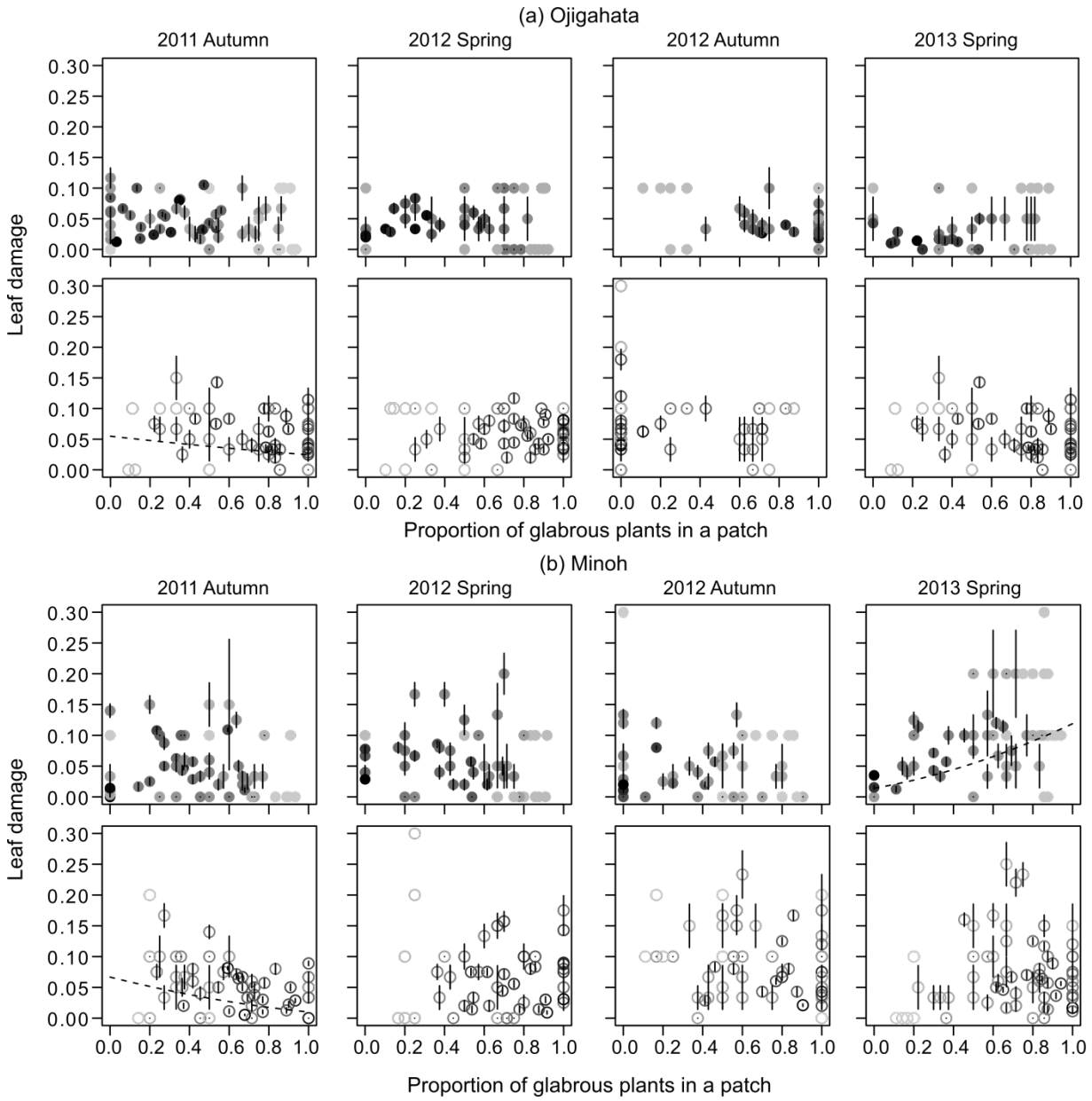
705 **Figure 4**



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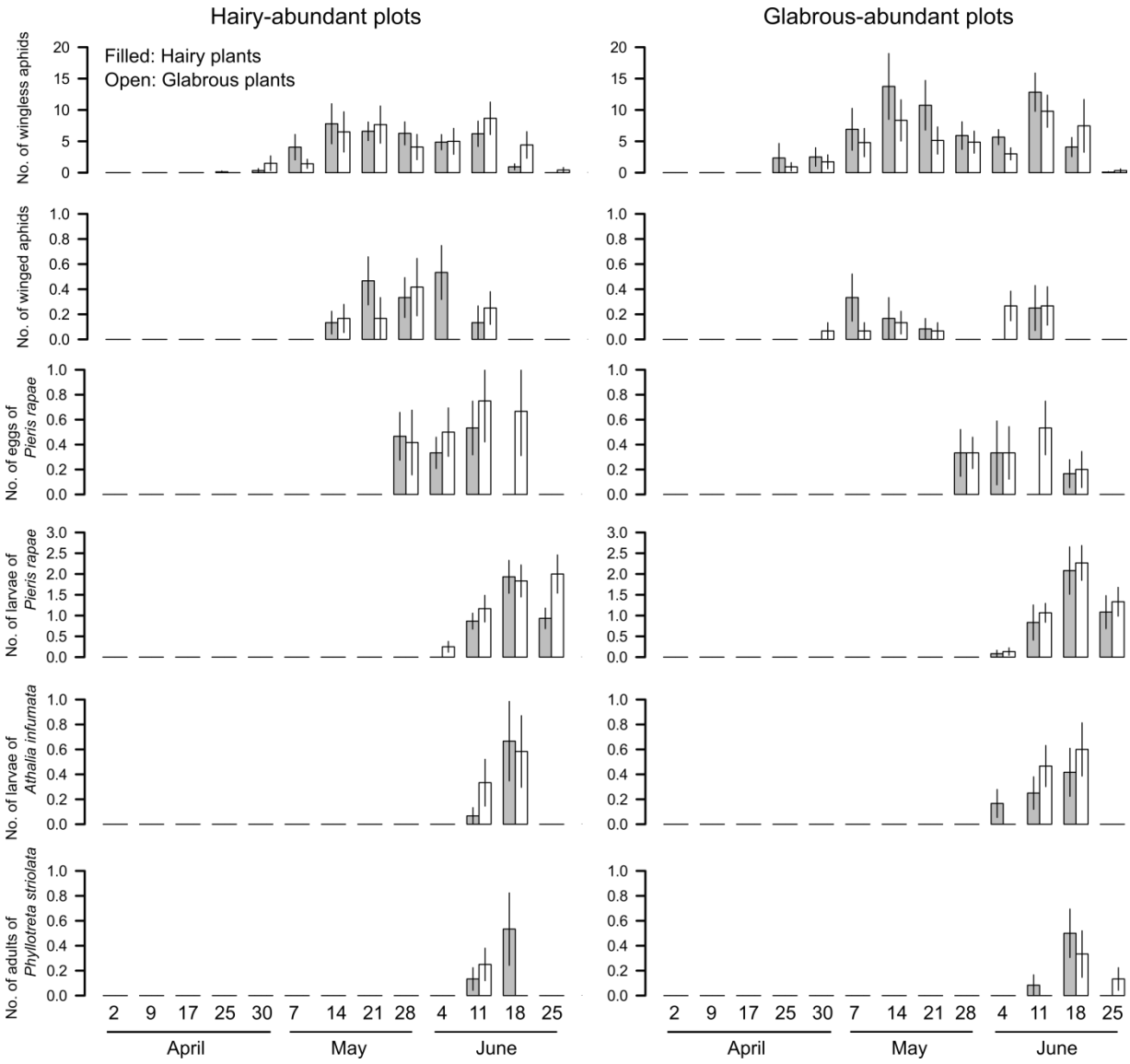
711 **Figure S1**



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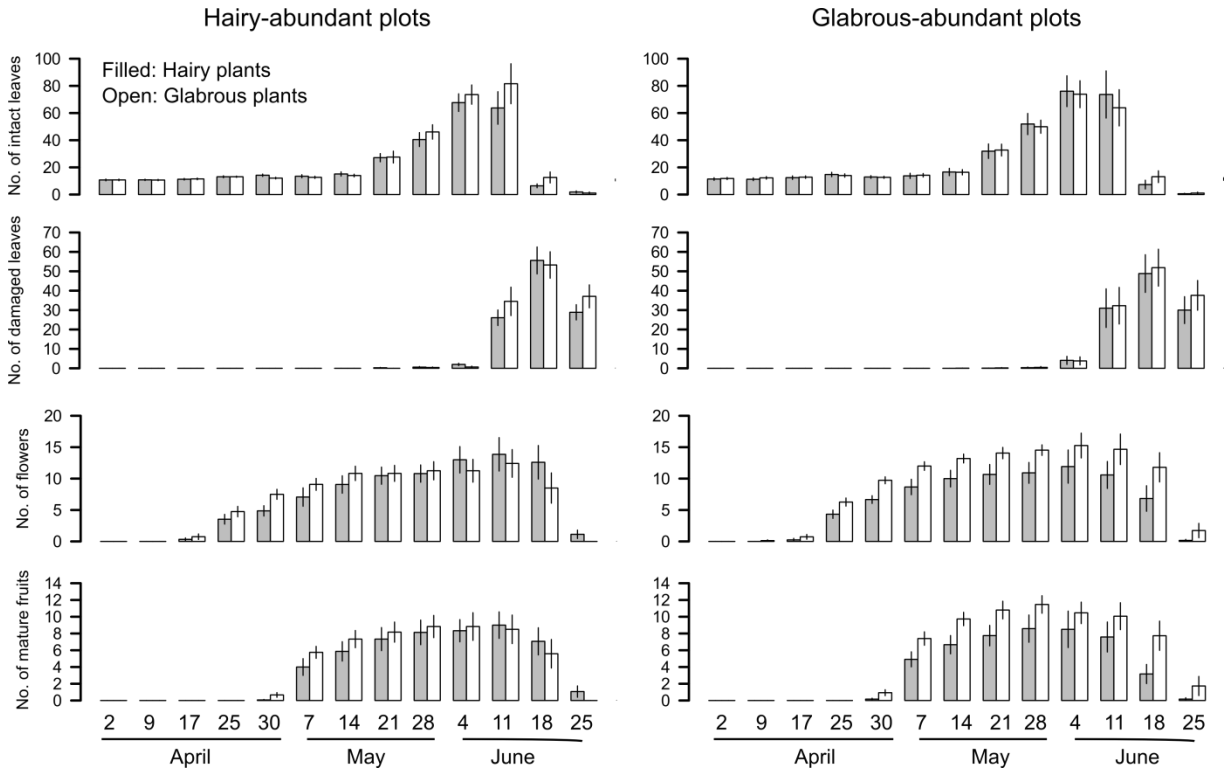
717 **Figure S2**



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